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TITLE OF THE INVENTION (500 characters max)

Proliferation of Precursor Cells

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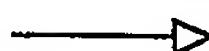
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Respectfully submitted,
SIGNATURE _____
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10 **PROLIFERATION OF PRECURSOR CELLS**

INVENTORS: Susan Meakin and Kathryn Volkening

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FIELD OF THE INVENTION

We disclose a method for *in vitro* proliferation of precursor cells and precursor cell progeny comprising exposing isolated precursor cells to culture, exposing the cell to culture medium containing a growth factor; inducing the cell to proliferate and maintain

5   proliferation of said cells by genetically modifying the cells *in vitro*; inducing differentiation of cells and using cells *in vivo*.

BACKGROUND OF THE INVENTION

Precursor cells are unique in their ability to proliferate for extended periods of time and

10   to differentiate under certain conditions into specialized cell types. Because of their distinctive characteristics, precursor cells offer unique opportunities to develop new therapeutic approaches for many diseases that are currently incurable. Precursor cell therapy is a developing field that aims to treat many degenerative diseases caused by the premature death or malfunction of specific cell types and the body's failure to replace or

15   restore them.

Precursor cell therapy approaches can be used to treat a wide range of human disorders, including types of cancer such as leukemia, neurological diseases such as Parkinson's Disease, Alzheimer's and ALS (Lou Gehrig's Disease), CNS damage, cardiac damage, liver damage and diabetes. Currently, the best treatments can only delay the

20   onset and relieve the symptoms of ill health caused by the disease. Although some diseases can be addressed with transplant surgery, not enough donors exist to treat all patients and, even when rare donors can be found, this is limited to specific tissues and organs and is very expensive. Many have looked to precursor cells as a possible alternative since they can be collected, grown and stored to provide a plentiful supply of

25   healthy replacement cells for transplantation into any body site using much less invasive surgery than conventional transplants. In addition to their plasticity, precursor cell can

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offer a non-immunogenic source of cells to avoid the problems of finding a compatible donor and transplant rejection by the immune system.

Traditionally, CNS disorders have been treated with pharmaceutical compounds with varying degrees of efficacy; however, there are a multitude of CNS disorders which

5 continue to be untreatable using conventional methods and compounds. Cell therapy approaches aim to replace defective cells instead of treating the disease effects. Possible approaches include using transplanted tissue to directly replace lost tissue, or implanting genetically engineered cells that secrete factors which promote survival and/or proliferation. Cell therapy using traditional primary cell replacement strategies involve

10 transplantation of exogenous tissue, and activation of proliferation of endogenous cells. However, scarcity of human material, the lack of proliferative capacity of primary cell cultures, and problems associated with the aberrant characteristics that immortalized cell lines often display identifies the need for a dependable source of precursor cells whose proliferation does not affect the phenotype.

15 Precursor cell technology promises the ability to produce a large number of cells efficiently in culture, and avoids many of the technical and ethical limitations associated with the use of primary embryonic tissue in current transplantation regimes.

Neural precursor cells exist in various regions of the CNS throughout the mammalian lifespan and there has been a growing interest in the characterization of

20 neural precursor cells for basic developmental biology studies, drug screening and for therapeutic applications to the damaged brain. Much research is currently focused on promoting endogenous neural reconstruction and using neural precursor cells in cell replacement or recruitment strategies for the treatment of a variety of human neurological conditions including Parkinson's disease, ALS, Huntington's disease, Alzheimer's,

25 multiple sclerosis and ischemic brain injury. Transplantation of neural precursor cells or their derivatives into a host brain represents a potential treatment for many

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neurodegenerative diseases, especially Alzheimer's and Parkinson's disease which are characterized by a continuous loss of specific populations of neurons associated with progressive loss of function.

5   Self-renewing neural precursor cells, with the *in vitro* capacity to produce all the major cell types of the brain, would be beneficial for transplanting. Since neural precursor cells could give rise to the principal cellular phenotypes comprising the mature CNS: neurons, astrocytes and oligodendrocytes they may also provide accessible model systems for studying neural development. In addition, because of their migratory behaviour, neural precursor cells also have the potential clinical applications as cellular

10   vectors for gene delivery and the expression of therapeutic proteins.

          While considerable recent progress has been made in terms of developing new techniques for the long-term culture of human precursor cells, the successful clinical application of these cells is presently limited by our understanding of precursor cell proliferation. The factors controlling neural precursor cell survival, undifferentiated state,

15   proliferation, and cell-cycle number are beginning to be identified, but the links between them remain unclear. In order to make precursor cell therapy viable, an effective method must be developed to collect, isolate and grow a plentiful supply of precursor cells that can meet stringent testing requirements for autoimmunity and compatibility. Therefore a need exists for novel methods to create a large standardized supply of precursor cells

20   suitable for cell therapy because culturing long-term precursor cell populations without differentiation has been difficult using current techniques and is limiting practical precursor cell applications.

          Many growth factors with stimulatory effects on the proliferation, differentiation and development of precursor cells have been identified. Many precursor cell culturing

25   techniques depending on the lineage, include one or more growth factors including Fibroblast Growth Factor (FGF), to help control cell fate. FGF and neurotrophin

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signaling pathways (including Nerve Growth Factor (NGF); Brain-derived Growth Factor (BNDF), Neurotrophin 3 (NT-3) and Neurotrophin 4/5 (NT-4/5)) are known to regulate in a coordinated manner many facets of development, including patterning, central nervous system development, and neural crest (NC) migration/proliferation.

5   We propose that FGF Receptor Substrate 3 (FRS3), an FGF and neurotrophin receptor adapter protein, can be used to successfully expand a population of precursor cells. In addition, by expressing FRS3, we can induce increased and/or continuous proliferation in precursor cells resulting in lower culture times and more rapid proliferation rates than normally seen in precursor cells.

10 **SUMMARY OF INVENTION**

The present invention relates to methods for culturing and proliferating precursor cells and the use of these cells and their progeny for therapeutic, research and drug screening purposes.

In a preferred embodiment of the invention, we disclose a method of stimulating and

15 maintaining precursor cell proliferation *in vitro* to produce an appropriate amount of precursor cells or precursor cell progeny to use for therapeutic, research and drug screening purposes.

In another non-restrictive preferred embodiment of this invention, we disclose a method of stimulating and maintaining precursor cell proliferation *in vitro* by

20 manipulating FRS3 in precursor cells to be responsive to growth factors including FGF and neurotrophins but not EGF.

In another non-restrictive preferred embodiment of this invention, we disclose a method of stimulating and maintaining precursor cell proliferation *in vitro* by transfecting precursor cells with FRS3 (under an inducible system) using retroviruses or lenti-viruses,

25 and monitoring and controlling the proliferation using the inducible system. In addition,

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precursor cells can be additionally genetically modified to produce therapeutically effective proteins.

In another non-restrictive preferred embodiment of this invention, we disclose a method of stimulating and maintaining precursor cell proliferation by manipulating the enhancer and promoter system for FRS3 and modulating endogenous FRS3 by providing the appropriate amounts of proteins and transcription factors.

In another non-restrictive preferred embodiment of this invention, we disclose a method of stimulating and maintaining neural precursor cell proliferation *in vitro* by manipulating FRS3 in precursor cells.

In another non-restrictive preferred embodiment of this invention, we disclose a method of stimulating and maintaining skin precursor cell proliferation *in vitro* by manipulating FRS3 in precursor cells.

In another non-restrictive aspect of this invention, we disclose a precursor cell line that contains an inducible FRS3 and can proliferate to produce the desired amount of cells when exposed to growth factors including Fibroblast Growth Factor or neurotrophins but not Epidermal Growth Factor.

In another non-restrictive aspect of this invention, we disclose a precursor cell line that is a neural, cardiac, hepatocyte or skin-derived precursor cell line.

In another non-restrictive aspect of this invention, precursor cells are proliferated in vivo for the treatment of disease.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

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BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1: A: Vector transcript expression in E9.0 through E15 hFRS3 transgenic animals
by RT-PCR. Controls include actin and murine FRS3. Human FRS3 and ß-galactosidase
are transcripts derived from microinjected constructs showing expression of the transgene
over this period of development. B: Fluorescent *in situ* hybridization for ß-galactosidase
mRNA in control (negative littermates) and hFRS3 transgenic embryos. Photos are
inversed so that signal is black; scale bar=100um; arrows=dorsal root ganglia; NT=neural
tube.

Figure 2: A: Hematoxylin and Eosin stained sections from liver (scale bar=50um), lung
and uterus (scale bars=100um) of adult control (CD1) and hFRS3 animals. *=bands of
myometrium extending through uterine lumen of hFRS3 transgenic animal. Note also the
small alveoli and thickened alveolar walls, and smaller hepatocytes in hFRS3 adults. B:
Measurements of the neural tube in E14 control (negative littermate) and FRS3 animals
at T4 level of the developing spinal cord (RP: roof plate; FP: floor plate; NE thick:
overall thickness of neuroepithelium; Ven: ventricular layer; Man: mantle layer; Mar:
marginal layer; NE diameter: overall diameter of neural tube; lumen: diameter of luminal
opening within neural tube; *=statistically different within groups).

Figure 3: A: Dorsal root ganglion duplications and triplications found in FRS3 animals at
E12 (arrows). Scale bar=100um. B: P0 longitudinal sections of dorsal root ganglia from
CD1 and FRS3 animals. Arrows show duplicated ganglia adjacent to nerve tract.
Arrowhead is a degenerating ectopic dorsal root ganglion structure lacking nerve tract

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support. Scale bar=200um. C: Toluidine blue stained E16 dorsal root ganglion showing large neuronal cells (arrows) and smaller diameter cells (arrowheads). Scale bar=50um.

Figure 4: A: Counts across three FRS3 over-expressing lines established from
5   independent microinjections compared to CD1 and negative littermates at E14 (*=statistically significant between groups). B: Dorsal root ganglion cell counts averaged across the three strains above between E14 and P0 (*=statistically significant within groups; FRS3 DG=ectopic duplicated ganglia seen in hFRS3 transgenic animals not associated with a nerve tract).

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Figure 5: *In situ* hybridization in embryonic dorsal root ganglia showing changes in transcript expression in FRS3 transgenic animals. Photos are inversed so that signal is black, scale bar=100um. Arrows point at dorsal root ganglia.

15   Figure 6: A: TrkA and TrkB *in situ* hybridization in a P0 hFRS3 transgenic dorsal root ganglion showing increased TrkB expression overlapping with TrkA expression in many cells (LM=light micrograph, dotted line denotes dorsal root ganglion outline). B: Ki67 immunohistochemistry to detect proliferating cells in the E14 dorsal root ganglion (arrows; positive signal is black). Scale bar=50um.

20   Figure 7: Frizzled immunohistochemistry in the E12 spinal cord of negative littermate controls and hFRS3 transgenic mice. Frizzled is a marker used to identify neuronal precursor cells in the developing nervous system. Note the increase staining in hFRS3 transgenic animals, widely spread throughout the dorso-ventral axis of the neural tube in comparison with ventral location in control animals.

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Figure 8: A: Fluorescent *in situ* hybridization (photos are inversed, signal is black) for sonic hedgehog, and immunohistochemistry for versican in control (negative littermates) and hFRS3 transgenic animals. B: Neural crest cell delamination in hFRS3 and control littermate E9 embryos in culture. Values are not statistically different. Scale bar=100um.

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Figure 9: A: Neural crest cell proliferation in hFRS3 and control E9 embryos at 72 hours in culture. Values are statistically different ($p < 0.00005$). B: Light micrographs of neural crest cells after 24 (10x) and 72 (20x) hours in culture. Note the increased cell number in FRS3 animals over controls at 72 hours.

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DETAILED DESCRIPTION OF INVENTION

This patent discloses a novel use of the FRS3 adapter protein to stimulate and maintain proliferation of precursor cells. Accordingly, the present invention provides a method of culturing the precursor cells, transfecting the precursor cells with an inducible FRS3,

15 causing the precursor cells to proliferate, preferentially terminating proliferation and inducing differentiation of the precursor cells for use in therapeutic, research and drug screening regimes.

Fibroblast growth factor (FGF) and neurotrophin signaling pathways are known to regulate in a coordinated manner many facets of development, including patterning

20 (Hama et al., 2001; Kusakabe et al., 2001; for review see Dono, 2003), central nervous system development (Ortega et al., 1998; Raballo et al., 2000; Vaccarino et al., 1999, for review see Dono, 2003), and neural crest (NC) migration/proliferation (for review see Dono, 2003). The FGF Receptor Substrates 2 and 3 (FRS2/SNT1/FRS2α, FRS3/SNT2/FRS2β) are small adapter proteins that link activated FGF receptors and the

25 Trk family of neurotrophin receptors to downstream signaling molecules. It has been

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previously shown that FRS2 is required for increased neuritogenesis, neuron survival (Hadari et al., 1998; Meakin et al., 1999) and differentiation. FRS3 shares similar structure to FRS2 but until now, the role for FRS3 has been unknown. Here we disclose a novel role of FRS3 in maintaining proliferation in neural-crest derived neural precursor cells without differentiation, and influencing the fate restriction of DRG-destined neural precursor cells as they form mature neurons, as well as a role in the expansion of neural precursor cells in the developing central nervous system. We have found that expression of FRS3 results in the maintenance of proliferation of precursor cells in transgenic mice, effectively increasing the population of these cells prior to differentiation.

FRS2 and FRS3 associate with the FGFR1 constitutively in a phosphorylation independent manner, while association with the neurotrophin receptors (Trk receptors) in response to NGF signaling requires receptor phosphorylation for interaction with FRS2 (Xu et al., 1998; Meakin et al., 1999; Easton et al., 1999; Dhalluin et al., 2000; Ong et al., 2000) and with FRS3 (Meakin and Dixon, unpublished data). Prevoius work has shown that FRS2 is required for neuron survival and differentiation (Xu and Goldfarb, 2001; Hadari et al., 1998). While FRS3 shares similar structure with FRS2, the specific role(s) of FRS3 are unknown.

Developmental studies show that FRS2 and FRS3 are both dynamically expressed during embryo development in the mouse, with FRS3 more spatially restricted than FRS2 (McDougall et al., 2001). FRS3 is expressed in several adult tissues, and in the embyro almost exclusively in the developing spinal cord, the ventricular layer of the neuroepithelium of the brain, and condensing somites. FRS2 is much more widely spread throughout the embryonic nervous system (McDougall et al., 2001), with expression throughout all layers of the developing neural tube (NT), brain and in dorsal root ganglia (DRG; unpublished observations). Spatial and temporal differences in expression of these two adapters point to different roles in embryonic development. The

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mouse gene knockouts of FRS2 revealed that FRS2 was required for some vital facet of development, without expression embryonic lethality occurred by E7-E7.5, although the reason behind mortality was not determined (Hadari et al., 2001). US Patent 6,310,181 claims the use of human adaptor protein FRS2 in the diagnosis, prevention or treatment

5   of abnormal conditions associated with cell proliferation or cell differentiation. No such role has been identified for FRS3 to date.

Here we report spinal NT and DRG phenotypes resulting from transgene over-expression of human FRS3 (hFRS3) under the second intronic region enhancer of the nestin gene and minimal TK promoter. These mice show gross abnormalities in the

10   DRG, including duplications and triplications in structures that form in more ventral locations to the normally located DRG. There is a statistical increase in the number of cells found in both normal and duplicated DRG beginning at E10 and persisting to E16. By P0, most duplicated DRG structures exhibit cell loss when compared with control DRG unless directly in contact with nerve tracts, in which case cell numbers remain

15   abnormally high. Ganglia that are formed in the normal location in FRS3 over-expressing animals show a marked increase in cell number over control DRG. We also show a reduction of sonic hedgehog (Shh) expression in the NT which correlates with the formation of ectopic DRG structures, providing evidence of a reciprocal interaction between FGF and Shh signaling and patterning systems. We highlight a new role for

20   FRS3 in the proliferation of precursor cells

The term "animal" as used herein includes all members of the animal kingdom, including humans. Preferably, the animal to be treated is a human.

The term "a precursor cell" as used herein means a cell that is pluripotent and capable of self-renewal and is capable of trans-differentiation into multiple tissue types

25   upon differentiation. The precursor cell can be obtained from a variety of sources including, but not limited to, neuronal tissue, peripheral blood, bone marrow, skin,

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umbilical cord cells (including umbilical vein endothelial cells) as well as embryonic cells. Preferably, the precursor cell is derived from adult bone marrow, adult skin or peripheral blood. The precursor cell can be derived from any animal and is preferably from a mammal such as a rodent or a human. The precursor cells used in the methods of

5   the invention may be patient derived (autologous) or from a donor (allogeneic). The precursor cells can be administered to the animal using a variety of techniques including systemically or directly at the site of a tissue or organ, such as the liver, pancreas, cardiac tissue, brain or spinal cord. The precursor cell may be administered intravenously or by portal vein injection. Administering an agent to a cell includes both *in vitro* and *in vivo*

10   administrations.

The term "a cell" as used herein includes a single cell as well as a plurality of population of cells. Administering a precursor cell includes administering cells that have been prepared or expanded *in vitro* as well as expanding or stimulating precursor cells that are present in the animal *in vivo*.

15   The term "treatment or treating" as used herein means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease

20   progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" can also mean prolonging survival as compared to expected survival if not receiving treatment.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result (e.g. to treat

25   Parkinson's Disease, Alzheimer's, injury, autoimmune disease, hyperglycemia, etc).

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The present invention also includes pharmaceutical compositions for use in admixture with a suitable diluent or carrier.

The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

A pharmaceutical composition may be administered in a convenient manner such as by injection (subcutaneously, intravenous, intramuscular, etc.), preferably directly at the site, or by oral administration, inhalation, or transdermal administration. The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration. The pharmaceutical compositions include, albeit not exclusively, the invention in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other biologically active agents.

Example Method

In Vitro Proliferation

Neural precursor cells are cultured *in vitro* with growth factors known in the art, including FGF. Using a retroviral or lentiviral system, neural precursor cells are infected with FRS3 under an inducible system. Neural precursor cells are then cultured with the inducer to stimulate and maintain proliferation of the neural precursor cells through the activity of the inducible FRS3 until desired amount of proliferation is reached.

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Proliferation

The amount of proliferation can be measured by cell counts and by taking a subset of cells, putting them in separate wells and performing BrdU incorporation and Ki67 immunohistochemistry on the cells.

Differentiation

The neural precursor cells can be tested to determine if they underwent differentiation while they were proliferating by testing for the presence and absence off precursor cell markers. Undifferentiated neural precursor cells should not express C-kit, GFAP, smooth muscle actin or neurofilament 160. In addition, the neural precursor cells should express the same markers after proliferation as they had originally expressed.

Transplantation

After proliferation, the neural precursor cells can be directly transplanted or further fated to form specific cell lines and be transplanted with a pharmaceutically acceptable vehicle into the organism with the CNS disease such as Parkinson's or Alzheimer's.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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EXPERIMENTS

Construct expression:

The transgene construct was designed to target neural tissue using the second intronic region enhancer of the Nestin gene, and under the minimal TK promoter in transgenic

5   CD1 mice. The reporter β-galactosidase (β-gal) gene was included with an upstream internal ribosomal entry signal for tracking gene expression. Lothian and Lendahl (1997) found that β-gal expression driven by a similar system involving the Nestin second intronic region enhancer was observed from E10.5 through E14 in neural progenitors in the central nervous system and earlier in NC. Yaworsky and Kappen

10   (1999) found that the second intronic region enhancer targetted expression of transgenes to the central nervous system, specifically to neural precursor cells. Using this system we expected to express hFRS3 in neural tissues (the developing neural tube, brain and neural crest) between approximately E9 and E14. Of 9 hFRS3 lines established, 3 were maintained for study, 2 of these lines are still maintained (designated 22P3 and 1I3),

15   while 1 line (designated 23B2) stopped breeding in the sixth generation (P5). Lines were maintained as heterozygous animals, as all homozygotic animals appeared lethal and were never carried to term. All data presented are representative of data from heterozygous animals from all three lines unless stated otherwise. Neural crest experimental data are derived from the 22P3 and 1I3 lines only.

20   To determine the timing of construct expression in embryonic development we examined hFRS3, β-gal and control (murine FRS3, actin) gene expression in hFRS3 transgenic E9 to E15 embryos by RT-PCR (Figure 1A). Transcripts for hFRS3 and β-gal were detected as early as E9.5 and persisted until E14. Earlier than E9.5 and later than E14, transgenic hFRS3 and β-gal transcripts were not detected by RT-PCR, while control

25   transcripts were. Similarly, *in situ* hybridization for β-gal transcripts indicated

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expression from E9.5 through E14, but not at E15 (Figure 1B). When embryos were stained with X-gal, there was no β-gal activity detected, and on subsequent sequencing of genomic DNA from the lines we determined that the β-gal gene had a one base pair insertion at the 5' end, resulting in transcript production without translation of full-length

5   active protein.

In situ hybridization results agree with those of Lothian and Lendahl (1997), who expressed β-gal under the same Nestin second intronic enhancer. Expression was limited to the ventricular zone of the NT of the developing brain and spinal cord between E9.5 and E14.5, with expression from E10 through E14.5 in the DRG as well. No expression

10   of the β-gal transcripts was seen outside the central nervous system and the DRG.

Histology:

Adult animals and embryonic day 14 (E14) embryos were examined histologically for any gross abnormalities. Adult FRS3 transgenic animals displayed gross morphological abnormalities in the lung, liver and uterus (Figure 2). Liver

15   phenotype indicated hydropic degeneration, commonly seen with hepatitis. Mice were tested for both parasitic and viral hepatitis and confirmed negative (data not shown). Lung tissue had small alveoli, but animals were not in apparent distress and were normally active, suggesting that this phenotype was asymptomatic. The uterus was grossly enlarged in diameter, while being truncated in length, resulting in a worm-like

20   appearance of the entire organ, and the lumen was divided into a series of chambers by bands of muscle extending across from a thin myometrial layer (Figure 2). Litter sizes were small (9 animals, $p < 0.05$ compared with CD1 controls). Ovulation was apparently normal, with 12-15 oocytes released from both ovaries at mating, while litter sizes were significantly smaller. At E7, implantation sites were examined with an average of 9

25   implantation sites per pregnancy. The number of resorbing embryos was not

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significantly different from CD1s (FRS3: 2 embryos in 85 pregnancies; CD1: 3 embryos in 85 pregnancies). These data clearly show there was a failure of a portion of embryos to implant, probably due at least in part to the change of uterine morphology.

Embryonic defects were found in the head, NT, DRG and several tissues. Head

5   phenotype was found only in homozygotic animals and will be described elsewhere (Volkening et al., in prep). Data discussed in this paper are from heterozygous animals and is representative of all three transgenic lines. In the spinal NT at E14, hFRS3 transgenic animals displayed statistical decreases in the thickness of the neural tube roof plate (p=0.0001), mantle layer (p=0.0022), overall thickness of the neuroepithelium

10   (p=0.0059), and a decrease in the width of the neural tube lumen (p=0.0364; Figure 2B). The ventricular layer of the neural tube was statistically wider in FRS3 over-expressing animals than in negative littermate controls (P=0.0012; Figure 2B). While spinal NT phenotype appeared minimal there were marked changes found in the DRG, and consequently focus our studies on the DRG.

15   The DRG exhibited duplications and triplications of entire DRG structures (Figure 3). Individual DRG structures appeared grossly normal. Ectopic DRG were situated ventrally and medially to the ventral portion of normally positioned DRG (Figure 3A). These duplicated DRG were similar in morphology to normal DRG in the dorsal-ventral plane and rostral-caudal axis between E10 and E14. Duplicated DRG were

20   present at most but never at all levels of the neural tube, most often duplications were seen in the trunk region between T2-T4 level, and triplicated DRG were most commonly seen in the low lumbar and high sacral regions (L10-S5). Ectopic DRG were rarely seen caudal to S5.

At P0 (day of birth), ectopic DRG persisted in most FRS3 over-expressing mice.

25   Those duplicated DRG that were located adjacent to main nerve tracts of normally placed DRG structures appeared to be persistent (Figure 3B), while those that were not adjacent

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to nerve tracts were very small and apparently decreasing in both size and cell number. At P0, normally situated DRG in duplicated structures with well-developed nerve tracts commonly exhibited altered morphology, appearing to be lobed or oddly-shaped compared to the more oval shape of controls. Nerve tracts commonly coursed between

5 these ganglia, contacting both ganglia, as opposed to contacting only one end of one ganglion (Figure 3B).

When examined at the cellular level it was found that the majority of cells populating E14 DRG of hFRS3 over-expressing mice were of small diameter neuronal phenotype. There was a small proportion of large diameter cells of neuronal phenotype present (10-12% of total cells, compared to controls with 15-16% of total cells) while the

10 remainder of the DRG was composed of small diameter cells (Figure 3C).

Cell counts

We chose to concentrate cell counts at the T4 level, as this level most often showed duplications in DRG, and to maintain consistency as numbers of neurons within

15 DRG change depending on spinal column level. Total cell counts were performed on single DRG structures and are presented as number of cells per single DRG structure (Figure 4A). All cells with visible nuclei were counted across serial sections through entire DRG structures and counts were adjusted as per the method of Abercrombie (1946) to reduce sampling error. Negative littermate controls were animals from the same litters

20 as experimental animals, tested by RT-PCR and negative for human FRS3 transcripts. Control animals (CD1) and negative littermates had 2287 ± 70 and 2464 ± 116 cells per ganglion (not statistically different and consistent with data by Berg and Farel, 2000), while each of the FRS3 over-expressing lines had significantly higher numbers of cells (3086 ± 59, $p<0.001$; 3368 ± 177, $p<0.001$ and 2986 ± 93, $p<0.01$; see Figure 4A). The 35-

25 50% increase in cell number appeared to be due to an increased number of small diameter

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cells. Large diameter cells present in CD1 and control littermate animals were not statistically different (366±11 (16%) and 370±17 (15%), respectively). The total number of large diameter cells in FRS3 over-expressing animals was consistent with controls (375±9, 356±26, and 361±15) resulting in a lower overall percentage of large diameter

5   cells per DRG (10.5%, 12% and 12% per line) due to the increase in gross cell number per ganglion. At P0, DRG of control animals contained 3514±461 cells, while in hFRS3 animals those DRG structures that were adjacent to nerve tracts had a significant increase ($p<0.001$) in cell number to 9019±168 cells. Those duplicated DRG structures at distance from nerve tracts in hFRS3 animals contained only 597±3.5 cells, significantly

10 less ($p<0.001$) than control DRG (Figure 4B). These data show that hFRS3 transgenic animals exhibit an increase in cell number in DRG, as well as an increase in small diameter morphology cells, suggesting a role for FRS3 in DRG development.

Extension of the developmental program:

The DRG are formed by neural crest (NC) cells that delaminate from the dorsal

15 neural tube and migrate to a ventrolateral position beside the NT by approximately E10 in the mouse. There are several ways that DRG abnormalities in hFRS3 transgenic mice could have arisen. First, changes in migration could have resulted in non-DRG-fated NC being recruited into populating the DRG resulting in increased cell number. Alternatively, NC fated to reside in the DRG may be proliferating while migrating or

20 after populating the DRG, resulting in the increase seen in cell number without recruitment of other NC populations. Another possibility is that there are an increased number of NC delaminating from the NT and coming to reside in the DRG. To address these possibilities, we have examined markers for NC during migration, assessed proliferation occurring in the DRG throughout embryonic development of these mice and

25 delamination of NC from the NT.

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To determine what effect FRS3 over-expression might have on the NC, several markers known to be expressed in NC during development were investigated for mRNA expression by in situ hybridization. Negative littermates and CD1 animals were examined in parallel with FRS3 over-expressing animals to provide controls. Early markers Snail,

5   Pax3, bone morphogenic protein (BMP) 2 and BMP4 are all known to be involved in the selection of the neuroepithelium that will delaminate as the NC and migrate off the tube (Serbedzija and McMahon, 1997; Le Douarin and Kalcheim, 1999; Nieto, 2001; Knecht and Bronner-Fraser, 2002; Villanueva et al., 2002). From E9.5 through E12, Pax3, Snail, bone morphogenic protein (BMP) 2 and BMP4 mRNA expression were examined and

10   found to be normal (data not shown). As Pax3 and Snail were unaffected, and phenotype manifested only in the DRG, we did not pursue the expression of any known signaling proteins involved in melanocyte formation as the populations forming melanocytes and DRG are reported to be different in development (Luo et al., 2003; Aoki et al., 2003).

As duplicated DRG structures may have arisen due to an over-population of NC

15   through increased or maintained proliferation, TrkC, FGFR1 and TGFβ1 were examined (Figure 5). These proteins are known to directly affect proliferation of NC cells, with TrkC and FGFR1 eliciting a proliferative effect while TGFβ1 is known to decrease proliferation (Murphy et al., 1994; Zhang et al., 1997; Sieber-Blum and Zhang, 1997; Sieber-Blum 1998). TrkC expression was found to be much higher at E10 and remained

20   high through E14. FGFR1 and TGFβ1 were both decreased from control levels at both stages. As FRS3 is over-expressed, the decreased expression of FGFR1 could be due to receptor down-regulation due to increased signaling via the FRS3 adapter protein. The increase in TrkC expression with decreased TGFβ1 strongly suggests that the increase in number of cells populating the DRG may arise through increased proliferation, which is

25   discussed further below.

20

With the apparent change in cell number and prevalence of small diameter cells in the DRG, we also chose to examine markers known to be expressed in fate-restricted DRG sensory neuron development, including neurogenin 1 (Ngn1) and neurogenin 2 (Ngn2), as well as the markers expressed by differentiated DRG neurons, TrkA and TrkB

5   (Figure 5). Neurogenin 1 expression is required for formation of small diameter TrkA positive cells of the DRG, while Ngn2 expression fate restricts to the TrkB positive lineage (Ma et al., 1999). If Ngn1 and Ngn2 are knocked out, failure of DRG development occurs (Ma et al., 1999). Each of these genes showed abnormalities in expression levels of mRNA. Neurogenin 2 expression should be finished by E11 (Ma et

10   al., 1999); however, levels in the FRS3 over-expressing mice were elevated at E12, persisted to E14, and then decreased completely by E14.5 (data not shown). Neurogenin 1 expression was decreased compared to controls, but persisted at low levels to E14.5 (normally Ngn1 expression ceases at approximately E13, with expression dependent on earlier Ngn2 expression; Ma et al., 1999). TrkA expression in the DRG was decreased

15   significantly, while TrkB expression was very high, detected in almost all cells. When examined closer, the small diameter cells accounting for the 35-50% increase in DRG cell population appeared to be positive for TrkB and not TrkA. Larger diameter cells were TrkB positive, as expected, as medium and large diameter neurons are reported to be TrkB expressing and the first population of cells to differentiate in the DRG (Farinas

20   et al., 1998). By E14-15 there should be a large proportion (approximately 40-45%) of small diameter cells that are TrkA positive (Mu et al., 1993). There were very few TrkA only positive cells in FRS3 over-expressing mice (10-20 cells per ganglion examined at E14) suggesting that either these cells are wrongly fated, or that the developmental program leading up to TrkA expression has been delayed in hFRS3 transgenic mice.

25   When examined at P0, DRG neurons were found to be TrkA positive, or TrkB positive, with many smaller diameter cells being TrkB positive (Figure 6A). Normally, small

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diameter cells are mainly TrkA positive, but in hFRS3 transgenic animals it appears that hFRS3 has an effect on the fating of these neurons to TrkB or that some other system has been affected by FRS3 and is itself producing a change in fate. Further study is required to determine which is the case.

5   With changes in FGFR1, TGFβ1 and Trk C expression, transcription factors Ngn1 and Ngn2, Trk receptor expression, as well as the persistent population of small diameter cells in DRG, we investigated the continuation of proliferation in DRG to determine if this event had also been extended into times when it should be completed. The antigen Ki67 is expressed only in actively proliferating cells and can be used to

10   determine the proliferative status of cells (Gerdes et al., 1983; Gerdes et al., 1984). When sections through the DRG and NT of control E14 animals were examined, Ki67 was present in the ventricular region of the NT in control animals and at E10-12 in DRG (Figure 6B). Embryonic stage 14 hFRS3 over-expressing animals showed many Ki67 immunoreactive cells in the DRG, as well as in the immediate surrounding mesenchyme

15   of the NT (Figure 6B) evidence that proliferation is still occurring in DRG of these animals at a level higher than controls. This supports in situ data suggesting maintenance of proliferation by FRS3, possibly via TGFβ1 and TrkC expression changes. Taken together, it appears that the increase in cell number in hFRS3 transgenic DRG is due to increased and prolonged proliferation of DRG-fated NC.

20   The Frizzled proteins are receptors for the Wnts, proteins involved in the patterning of the central nervous system. Frizzled is known to be expressed in the developing central nervous system, in the ventral neural tube in the mouse by E11.5. The cells that are positive for Frizzled were found to be cells that are limited to neural restricted precursor cells (Van Raay et al., Dev Genes Evol 211:453-457, 2001). We

25   investigated the expression of Frizzled in the central nervous system of hFRS3 over-

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expressing mice and negative littermates to determine if over-expression of hFRS3 could induce an expansion of neural restricted precursor cells (as evidenced by an increase in Frizzled immunoreactivity). We demonstrated that an increase in Frizzled positive cells in the ventral neural tube due to the over-expression of hFRS3 (Figure 7). We found a

5 drastic increase in the immunoreactivity of the entire axis of the developing spinal cord and developing brain (brain data not shown) up to and including E14. Representative sections are shown in the attached figure-positive cells are brown in colour from the DAB substrate reaction. Surprisingly, expression was not restricted to the ventral region of the neural tube as expected, extended throughout most of the dorso-ventral axis of the neural

10 tube, seemingly excluding only the region of the roof plate and extreme dorsal neural tube. Increased Frizzled positive cells in hFRS3 transgenic animals over controls shows an expansion of the neural restricted precursor cell population within the neural tube. We show that these precursors are displaced or have migrated throughout the tube during development and likely include the entire population of cells responsible for neurogenesis

15 throughout all layers of the developing cord and brain.

Neural crest migration in hFRS3 mice:

In studies performed in Shh knockout mice, DRG phenotypes were seen that closely paralleled the phenotype seen in hFRS3 mice. With published reports of the complex interplay between Shh and FGF signaling, we chose to examine the expression

20 of Shh. Sonic hedgehog is normally expressed in the ventral neural tube and notochord (for Shh review see Marti and Bovolenta, 2002). In hFRS3 mice, Shh expression in the ventral neural tube was decreased over control animals at E10 (Figure 8A), but returned to normal levels by E14.5. A secreted form of Shh is known to elicit the production of versican family proteoglycans in surrounding tissues which are at least partly responsible

25 for directing NC migration in the head by limiting cell mobility (Feltsova et al., 2003). Due to the decrease in Shh expression, and the fact that the versican expression appears

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to be controlled by long-range Shh signaling, immunohistochemistry for versican was performed. We found that there was a marked reduction in the amount of versican surrounding the ventral portion of the NT in hFRS3 mice from E10 through E14. At E14.5, versican staining appeared to return to normal levels, comparable with control

5   littermate and CD1 embryos (Figure 8A). Immunoreactivity for versican appeared to be reduced but not absent between the medially portion of the ventral NT and the developing vertebrae, consistent with our observations that ectopic DRG structures were never found directly in the midline of the embryo, unlike reports of ectopic trigeminal ganglia formation in Shh knockout mice (Fedtsova et al., 2003).

10   Neural crest cultures

Neural crest cultures were prepared from E9 negative and hFRS3 expressing littermates, and cultured for 72 hours. We examined the possibility that there were more NC delaminating from the NT in FRS3 animals, which could account for the increased number of cells apparently residing in the DRG. The total number of cells delaminated

15   and the length of the NT were correlated to give the number of cells delaminating per millimeter of tube. The results showed that there was no statistical difference in the number of NC delaminating from the NT of hFRS3 over-expressing mice (333±52) or their control littermates (341±39; see Figure 8B) suggesting that there is no change in the process of delamination in hFRS3 transgenic animals and that extra NC delamination

20   from the NT was not causing the increase in cells ultimately residing in the DRG.

Cells were counted again at 72 hours, and then stained with X-gal. There was a slight increase in cell number per NT length in negative littermate controls from 333±52 cells to 381±50 while hFRS3 NC preps contained 560±45 cells when correlated with tube length. At 72 hours, there were on average 62±24 (19%) X-gal positive cells in negative

25   littermate dishes compared with 160±44 (29%) in FRS3 littermate cultures. Cells at 24

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hours have not been X-gal stained, but as delamination is occurring prior to the onset of expression of the construct, and expression of Snail, Pax3, BMP2 and BMP4 were normal, we expect there to be no difference between control and FRS3 transgenic embryos. The increase in X-gal positive cells at 72 hours represents a 2.6-fold increase in

5   cells expressing our construct in hFRS3 transgenic animals over 3 days in culture. In adult DRG from hFRS3 transgenic animals show a 2.6-fold increase in cell number in ganglia that persist on nerve tracts. Taken together these data strongly suggest that the increase in number of cells in DRG are due to the increased proliferation of a subset of NC cells that continue to proliferate after delamination has occurred. It also suggests that

10   the crest are at least partially pre-determined to become DRG within 72 hours of culture as not all NC are X-gal positive at this time. This also shows that expression of FRS3 under the second intronic enhancer of the Nestin gene is capable of targeting a subset of NC cells destined for the DRG and not all NC cell populations.

We have generated mice that over-express hFRS3 under the Nestin second

15   intronic region enhancer and minimal TK promoter. When using this system phenotypic abnormalities were introduced into these mice. We show over-population of DRG structures in FRS3 over-expressing animals, formation of ectopic ganglia, with maintenance of ectopic ganglia in contact with nerve tracts later in development demonstrating a central role for signaling via FRS3 in these processes.

20   There are several possible ways that over-population of the DRG and ectopic ganglia could occur: First, there may be increased delamination and thus migration of greater numbers of NC to ectopic locations after proliferation has occurred. Second, NC could arrive at ectopic locations and proliferation continues to produce a ganglion of abnormal size. Third, a combination of these two processes may be responsible. Our

25   current study shows proliferation in NC during migration, as well as in formed DRG as late as E14, suggesting that proliferation is prolonged and occurring within DRG after

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they are established. Other trunk structures formed from NC such as the sympathetic and enteric ganglia appeared normal in hFRS3 animals, strongly suggesting that recruitment from these NC was not occurring to add to the population destined for DRG, but that extra population was derived from proliferation or increased delamination from the NT.

5   We have ruled out increased NC delamination from the NT, and have shown evidence of increased proliferation of NC cells. These data clearly demonstrate an increase in proliferation of DRG-fated NC. In older animals, DRG that are not supported by nerve tracts degenerate, strongly suggesting that these ganglia form in completely ectopic locations, and are unable to establish trophic support from targets. Those structures able

10   to establish nerve tracts appear to have high cell survival and persist with increased cell numbers. Fibroblast growth factor signaling is known to promote proliferation of NC (Murphy et al., 1994; Zhang et al., 1997; Sieber-Blum and Zhang, 1997; Sieber-Blum, 1998) which agrees with our findings in hFRS3 transgenic mice having more cells populating the DRG. For the population of NC which will form the DRG, proliferation is

15   thought to be β-FGF driven, requiring FGFR1 to be expressed on migrating cells and β-FGF presence in the environment with expression of a neurotrophin (Murphy et al., 1994; Zhang et al., 1997). Up-regulation of TGFβ1 results in negative regulation of FGF-mediated proliferation (Zhang et al., 1997). Concomitant with this is expression of receptors allowing sensitivity to select neurotrophins, which results in differentiation into

20   either TrkA, B or C positive cells depending on their fate restriction (Murphy et al., 1994; Sieber-Blum, 1998). FRS3 is known to interact with FGF-activated FGFR1. By over-expressing FRS3 we show that you can increase FGF signaling in these cells without increasing FGFR expression, producing maintenance of the proliferating state for a period of time that corresponds with construct expression. Over-expression of FRS3 in

25   the NC population while migrating and shortly after DRG condensation would account

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for the increase in cell number seen in DRG from FRS3 over-expressing animals. The decrease in TGF-β in hFRS3 animals shows there is also down-regulation of inhibition causing maintenance of proliferation. Delineating a role for FRS3 in the maintenance of proliferation.

5   We have also shown that in hFRS3 over-expressing mice there is a clear decrease in Shh expression with concomitant changes in Ngn expression suggesting that all these pathways are linked, supporting data by Ota and Ito (2003). Perez et al. (1999) have shown that migrating neural crest expressing Ngn2 appear committed to sensory neuron fate when exposed to BMP2. Neural crest cells in hFRS3 transgenic mice express Ngn2

10 much longer than normal, have normal levels of BMP2 and would therefore contribute to sensory DRG neurons. Coupled with decreased versican expression, ectopic DRG structures are found each exhibiting increased cell numbers over controls.

      It has been shown in trigeminal ganglia that the expression of FGF2, and signaling via FGFR1 (which is known to interact with FRS3), can cause the decrease of

15 Ngn expression (Ota and Ito, 2003). Sonic hedgehog expression has no effect on Ngn2 expression in the same system (Ota and Ito, 2003) but appears to be required for Ngn1 expression. These observations are consistent with hFRS3 over-expression causing decreased Ngn1 expression, but not decreasing Ngn2 expression if in fact Shh is involved in the Ngn1 expression cascade. As hFRS3 transgenic mice show a decrease in Shh, we

20 would expect to see decreased Ngn1 expression that would return to normal upon Shh returning to normal at E14.5. Neurogenin 2 expression is prolonged until E14.5 in transgenic animals, where in normal animals Ngn2 expression decreases by E11 (Ma et al., 1999). There is an interplay between Ngn2 and Ngn1 expression, with Ngn2 expressed earlier (E9-E10.5) in the mouse embryo, and Ngn1 expression requiring Ngn2

25 expression (Ma et al., 1999). In addition, Ngn1 expression has been shown to also require Shh expression in the trigeminal ganglia, while Ngn2 expression occurs independent of

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Shh (Ota and Ito, 2003). Human FRS3 transgenic mice exhibit an abnormally long period of Ngn2 expression, with decreased Ngn1 expression, both which appear to return to normal upon transgene inactivation. Sonic hedgehog expression is clearly down-regulated in these transgenic mice, which would agree with the delay in Ngn1 expression

5   while Ngn2 expression is present.

Expression of the construct persists until E14, after which time hFRS3 expression is no longer detected. This decrease in FRS3 is concurrent with an increase in Shh expression. This evidence also strongly suggests that hFRS3 expression has an effect on the expression of Shh, as Shh is up-regulated to normal levels upon cessation of construct

10   expression. Multiple proteoglycans have been implicated in the control of NC migration, including aggrecan and collagen IX (Ring et al., 1996; Perissinotto et al., 2000; Perris et al., 1996) and direct interactions between the hedgehog family and proteoglycans have been shown in tooth development (Gritli-Linde et al., 2001).

Human FRS3 over-expressing mice did not display single DRG structures

15   located in the midline, as would be expected if all versican expression was affected. There are two main locations of Shh expression in the embryo, the ventral neural tube, and the notochord (for review see Marti and Bovolenta, 2002). Versican immunohistochemistry does show a low expression of versican located in the midline of hFRS3 transgenic animals, ventral to the neural tube in the region that correlates with the

20   notochord. Expression of hFRS3 in the neural tube appears to have decreased Shh expression only in the neural tube, while the notochord expression is still normal and able to elicit versicans in this area. This would prevent migration of NC into the midline, but still permits ectopic laterally located ventral DRG. This is consistent with expression of the construct. We have used the Nestin second intronic region enhancer so expression is

25   limited to NT (Lothian and Lendahl, 1997). Taken together, the decrease in Shh signaling appears to be involved in the final location of ectopic DRG seen in these embryos.

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We have shown that by over-expressing FRS3, an adapter protein involved in signaling from the FGF receptor family, that we can influence the number of cells populating the DRG. We have also shown an effect on versican expression, which correlates with the formation of ectopic DRG seen in these mice. Ectopic DRG persist

5 past birth provided contact with nerve trunks was established. Over-expression of hFRS3 also leads to increased proliferation, and an extension in developmental program in DRG-fated NC cells, the latter of which manifests in alterations of cell fate. Taken together, these data demonstrate that FRS3 functions in the maintenance of proliferation early in sensory neuron precursor cells, and additionally, later in development can influence the

10 fating of these cells during differentiation.

Materials and Methods

Transgenic mice

Full length human FRS3 cDNA was derived from amplification of a human

15 cDNA library (Clontech), subcloned into pcDNA3.1-myc-his first, then excised and subcloned into pIRES2-EGFP. NcoI digest produced a fragment containing full length FRS3, myc tagged with IRES on the 3' end, which then was subcloned into Nestin-TK (gifted from Dr. McMahon, Harvard University) to give the final microinjection cassette. Transgenic mice were derived by pronuclear injection of linearized Nestin-TK-hFRS3-

20 LacZ constructs into CD1 mice (Robarts Barrier Facility, Robarts Research Institute, London, Ontario, Canada). Animals were assayed for the presence of human FRS3 by genomic PCR from tail sample, and RT-PCR of embyronic brain RNA with primers specific to the human gene sequence DNA (primers: forward: AGC CAC CCA ATG CTC TAG; reverse: GTG GGG GCA GGT TCT CAT AGT GCG). Of 9 lines

25 established, 3 were found to be germline transmitting and expressed hFRS3 when examined by RT-PCR. These lines were maintained for study. Each line was established

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from a single founder animal, and all lines have been maintained as heterozygous animals. No homozygotic animals were found on any lines, as tested by matings with CD1 animals and assaying all embryos for the presence of the gene. Homozygotic animals were determined to be lethal at approximately E9-E9.5 (Volkening et al., in

5   prep). Litters were tested from each breeding pair to confirm germline transmission and RNA expression. All animals carried to term were screened for hFRS3 genotype by tail sampling and amplification with hFRS3 specific primers (Volkening et al., in prep). All data in this paper are presented from heterozygous animals.

Animals were also produced by microinjection of the above cassette without the

10   hFRS3 coding region to produce Nestin-TK-LacZ lines. These animals were bred to homozygousity, and cross-bred to heterozygous hFRS3 transgenic mice to supplement the inactive LacZ coding found upon sequencing of the hFRS3 lines for neural crest experiments.

Ovulation, implantation sites and embryo loss

15   Female transgenic animals were mated with either CD1 or transgenic males from the same line. Two days after vaginal plug females were euthanized by carbon dioxide inhalation, and ovaries, oviducts and uterii dissected. Ovulation sites were counted on the surfaces of both ovaries. Embryos were flushed from oviducts and upper uterine horns with warmed PBS, counted and compared with number of ovulations. For

20   determination of implantation sites, ovaries and uterii were removed from plugged females at E7, focal uterine enlargements counted and dissected to confirm embryo presence and compared to the number of corpora lutea present on both ovaries.

Transgene expression analyses

Total RNA was extracted from E12 or E14 embryo heads by TRIzol (Invitrogen)

25   as per manufacturer's protocol. Poly(A) RNA was digested for 1 hour with 1 unit RNAse-free DNAse (Invitrogen), re-extracted with phenol:chloroform:isoamyl alcohol

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and reverse transcribed using Superscript RT (Invitrogen) as per manufacturer's protocols. Amplification of the resulting cDNA was performed as described above with primers for hFRS3, actin, β-galactosidase or mFRS3. Primers are listed in Table 1.

X-gal staining

5   Embryos were examined for X-gal staining as follows: Embryos were dissected from maternal membranes and fixed for 1 hour in 4% paraformaldehyde pH 7.2. Embryos were then rinsed 3 times for 15 minutes each in rinse buffer (5 mM EGTA, 0.01% deoxycholate, 0.02% NP40, 2 mM MgCl2), then incubated in stain solution (rinse buffer plus 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6 and 1mg/ml X-gal) for 12-15 hours at

10   37oC. Stained embryos were then rinsed in PBS, dehydrated, embedded in paraffin wax and sectioned at 5 um thickness in the transverse plane. Sections were dewaxed to PBS, counterstained with eosin, washed twice in 70% ethanol, rinsed in PBS, coverslipped with aqueous mounting medium (Geltol; Immunon) and photographed with an Olympus microscope under normal light.

15   In situ hybridization

In situ hybridization was performed as described in McDougall et al (2001) for neurogenin 1, neurogenin 2 (Ngn1, Ngn2; constructs were generous gifts from Dr. D.J. Anderson, Howard Hughes Medical Institute, Caltech, CA, USA), Sox10, Pax3, bone morphogenic protein 2 (BMP2), BMP4, Shh, FGFR1, TrkA, TrkB, TrkC, transforming

20   growth factor beta-1 (TGFβ1), Snail, and FRS3 (250bp coding region fragment of FRS3). Fragments for Sox10, BMP2, BMP4, FGFR1, TrkA, TrkB, TrkC, Shh and TGFBβ1 were derived from PCR of cDNA from E10-E16 embryos, Snail and Pax3 fragments were amplified from an E8 mouse cDNA library (Clontech). Primers are listed in Table 1 for all genes amplified. Amplicons were TA-cloned into pGEMT-Easy (Promega),

25   confirmed by sequencing for both sequence integrity and orientation of insertion and

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probes in vitro transcribed using SP6 or T7 RNA Polymerase (MBI Fermentas) as per manufacturer's specifications. All riboprobes were fluorochrome tagged with either Texas Red-UTP (Amersham) or Flourescein-UTP (Amersham).

Embryos from E9 to E14 were dissected free from maternal membranes, and one

5 forelimb removed for DNA analysis by PCR. The remainder of the embryo was fixed in 4% paraformaldehyde for 1 hour at 4 degrees celcius. Embryos were then dehydrated to 95% ethanol, and paraffin embedded using a Leica Tissue Processor. Embryos were serial sectioned at 5 um thickness in the transverse plane. Sections were then subjected to in situ hybridization as described in McDougall et al. (2001).

10 Histology

Embryos between E9 and E16 were dissected from maternal membranes, fixed, embedded and sectioned as above. For P0 pups (day of birth) sections of the spinal cord were cut between T1 and T5, fixed, embedded and sectioned as for embryos. Slides were stained with either Hematoxylin and Eosin (Fisher Scientific), or toluidine blue (Fisher

15 Scientific). Entire neuronal phenotype populations of DRGs were counted from at least 5 positive over-expressing animals from 3 lines of FRS3 animals and from 5 CD1 animals. Counts were performed on all DRG between T2 and T4 of the spinal cord. Counts of all DRG structures were kept separate for each DRG structure, even if several DRG structures were apparent at the same vertebral level in FRS3 over-expressing animals.

20 All cells with a visible nucleus in every section were counted, and all sections in each DRG were counted. Counts were adjusted as per the method of Abercrombie (1946) to reduce sampling error by counting cells with nuclei in adjacent sections. Analysis of variance statistical test was performed using InStat software to determine significance.

Hematoxylin and eosin staining was performed at room temperature. Slides were

25 dewaxed to PBS, stained in Weigert's Hematoxylin (Fisher Scientific) for 5 minutes,

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rinsed 10 minutes in running water, dipped 5 times in Eosin (Fisher Scientific), cleared in 95% ethanol and coverslipped with permount.

Toluidine blue staining was performed at room temperature. Slide were dewaxed to PBS, dipped 3 times in 1% toluidine blue (in PBS), washed 5 minutes in running

5 water, and coverslipped with Geltol (Immunon).

Ki67, Versican and Frizzled immunohistochemistry:

All steps were performed at room temperature until otherwise stated. For Ki67 immunohistochemistry: Sections were dewaxed to 95% ethanol and digested for 10 minutes in 3% hydrogen peroxide in methanol. Sections were then washed twice in

10 water, and blocked for 1 hour in 5% goat serum in PBS. A 1:50 dilution of Ki67 antibody (BD Biosciences) in 5% goat serum was incubated on cells overnight at 4oC. Slides were washed twice in PBS, 1:50 dilution of biotylinated IgG secondary antibody (in 5% goat serum) incubated for 30 minutes, washed twice in PCS and incubated by 1:50 dilution of streptavidin-HRP (in 5% goat serum) for 30 minutes. After washing in PBS, DAB

15 staining was performed with a Vecta-stain kit (BD Biosciences) as per manufacturer's protocol. Slides were washed in water, counterstained in hematoxylin, dehydrated to xylenes and mounted with permount (Sigma). Sections were photographed with an Olympus microscope with Hoffmann-contrast and a green daylight filter to enhance contrast between the DAB precipitate and tissues (precipitate appears dark brown-black

20 while tissues appear blue-green). For versican immunohistochemistry: Slides were dewaxed and rehydrated to PBS then digested for 30 minutes at 37 degrees celcius with 0.5 units chondroitinase (Sigma, resuspended 0.5 units/ml in 0.02% bovine serum albumin fraction V, 50 mM Tris pH 8.0 and 60mM sodium acetate), washed in PBS and blocked for 30 minutes in 5% goat serum at room temperature. Rabbit anti-versican

25 (Chemicon International) was diluted to 10ug/ml in blocking buffer and applied to slides overnight at 4 celcius. Slides were washed twice in PBS, then incubated for 1 hour in

33

horseradish peroxidase conjugated goat-anti-rabbit secondary antibody for 1 hour at room temperature. Following two PBS washes, DAB staining was performed with a Vectastain kit, and slides coverslipped as above.

The anti-Frizzled antibody was from Santa Cruz Inc. Paraffin sections of 5 um

5   thickness were obtained from E10 through E14 mouse embryos, both negative littermates and positive hFRS3 embryos (confirmed by PCR of limb bud DNA as described in Volkening et al., Genes Dev., in prep). Sections were de-waxed to PBS and immunohistochemistry performed as follows: Dewaxed and rehydrated sections were blocked for 30 minutes in 5% goat serum in PBS. Primary antibody (rabbit anti-Frizzled)

10   was used at 10mg/ml in blocking buffer overnight at room temperature. Sections were washed in PBS twice for 10 minutes each at room temperature. Secondary antibody (Goat anti-rabbit-horseradish peroxidase conjugate) was incubated on slides in blocking buffer for 1 hour at room temperature. Slides were washed twice in PBS and subjected to the DAB colour reaction as above.

15   Neural Crest Cultures

Heterozygous hFRS3 transgenic females were mated with homozygotic LacZ transgenic males and E9 embryos were dissected free from maternal membranes and placed in warmed PBS. Neural tubes were removed by careful dissection with 25.5 gauge needles and all surrounding tissues removed including somites and overlying

20   ectoderm. Individual tubes were placed in 4-well dishes (Nunc), one tube per well, and cultured overnight in neural crest medium (as described in Stemple and Anderson, 1992). Plates were coated with fibronectin (Sigma). After 12 hours, tubes were photographed and measured using a micrometer, then tubes were removed from culture using fine forceps and scraping with a 25.5 gauge needle. All cells of neural crest morphology were

25   counted after tubes were removed. All embryos were genotyped by PCR for hFRS3 as described above from remaining tissues after neural tube dissection. Genotyping was

34

completed after counts from culture, so that it was unknown which wells contained hFRS3 neural tubes to prevent any biases in counting. All embryos from three separate litters from two lines of hFRS3 mice were examined.

CLAIMS

5   1. A method of stimulating and maintaining the proliferation of precursor cells in an undifferentiated state by modulating the activity of the FRS3 adapter protein.

2. A method of according to claim 1 wherein FRS3 is modulated by transfecting the precursor cells with an inducible FRS3 using a virus.

3. A method according to claim 1 and 2 wherein the precursor cell proliferation is

10   controlled under the appropriate enhancer and promoter.

4. A method according to Claim 1,2 and 3 wherein the precursor cell population is directed to the appropriate cell fate and used for therapeutic purposes

5. A method according to Claim 4 wherein the therapeutic purposes is for human disorders that would benefit from cell replacement therapies.

15   6. A method according to Claim 1-3 wherein the precursor cells are proliferated for use in drug screening assays.

7. A method according to Claim 1-6 wherein the precursor cell is a neuronal precursor cell.

8. A method according to Claim 1-6 wherein the precursor cell is a skin-derived

20   precursor cell.

9. A method according to Claim 1 wherein the precursor cells are proliferated *in vitro* culture.

10. A method according to Claim 1 wherein the precursor cells are proliferated *in vivo*.

25   11. A method according to Claim 2, 4, 5 wherein the precursor cells are also genetically modified to produce therapeutic proteins.

35

12. A precursor cell line with an inducible FRS3 that is responsive to growth factors including fibroblast growth factor but not epidermal growth factor.

13. A cell line as in claim 12 that is used for drug screening, research and development or therapeutic purposes.

5

36

A

B
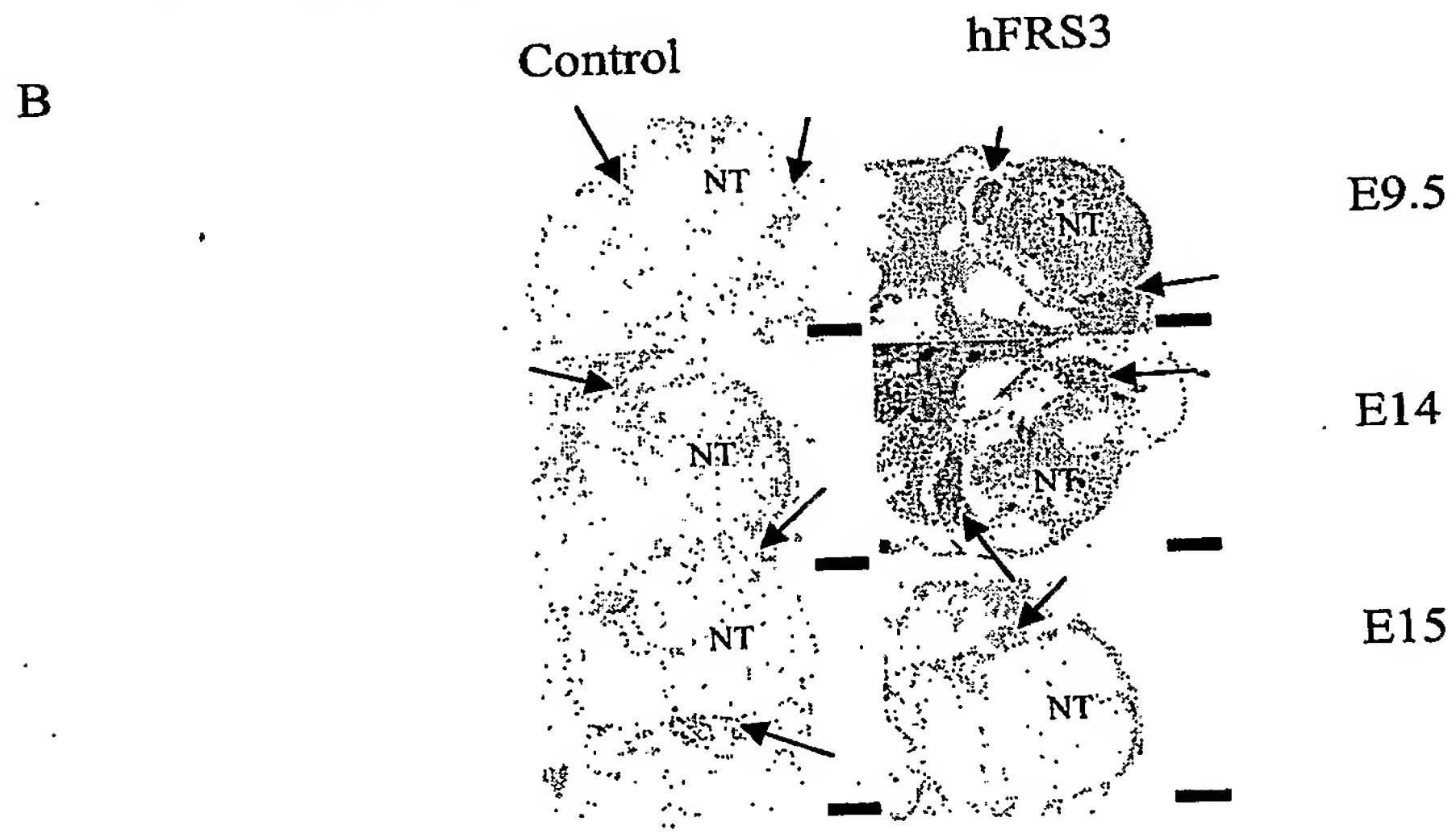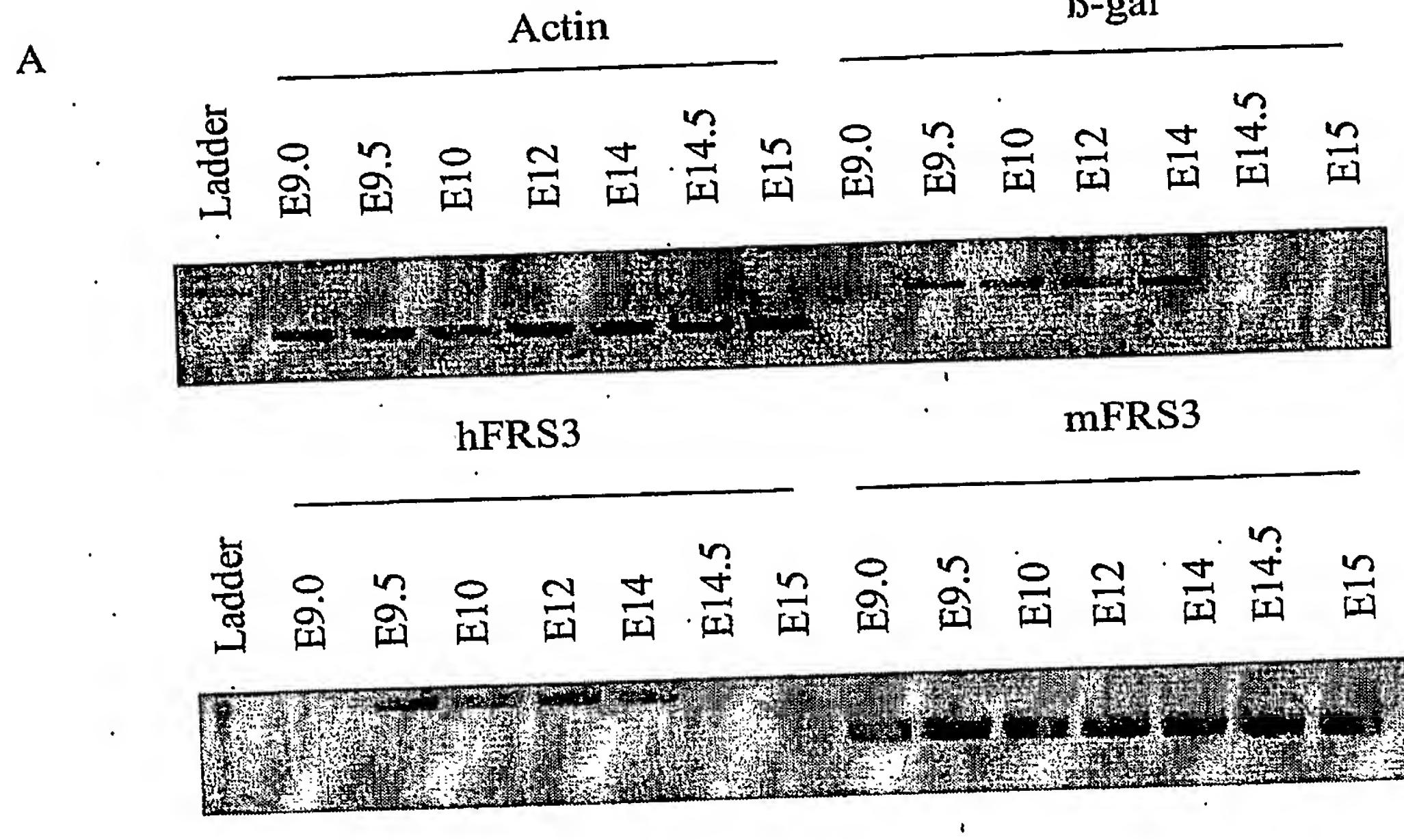
Figure 1

A

Liver

Lung

Uterus

Control littermate   hFRS3

B

Neural Tube measurements at E14

Region of Neural Tube

C

Neural Tube measurements at E14

Region of Neural Tube

Figure 2

A — CD1 control · FRS3-1I3 · FRS3-22P3 · FRS3-23B2

B — CD1 · FRS3-22P3

C — Control · FRS3

Figure 3

A Number of cells per dorsal root ganglion at E14

B Number of DRG Neurons over development in hFRS3 over-expressing and control mice

Figure 4

Figure 5

TrkA   TrkB

A

LM

B   Control   hFRS3
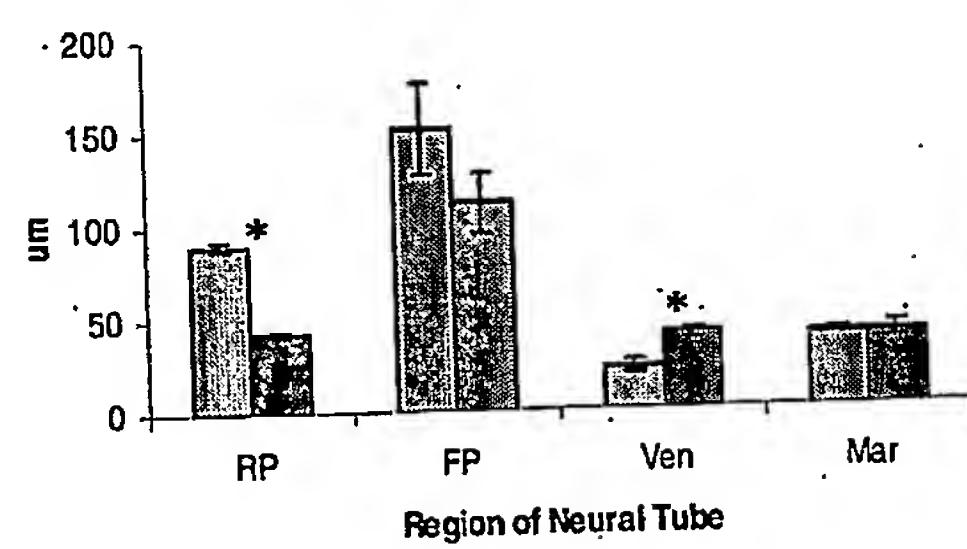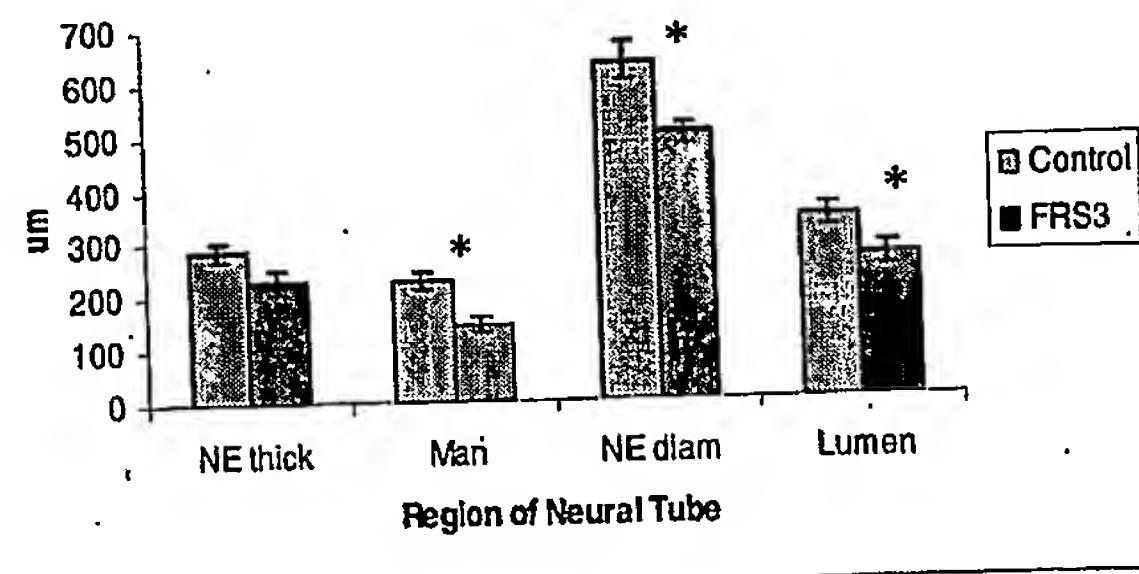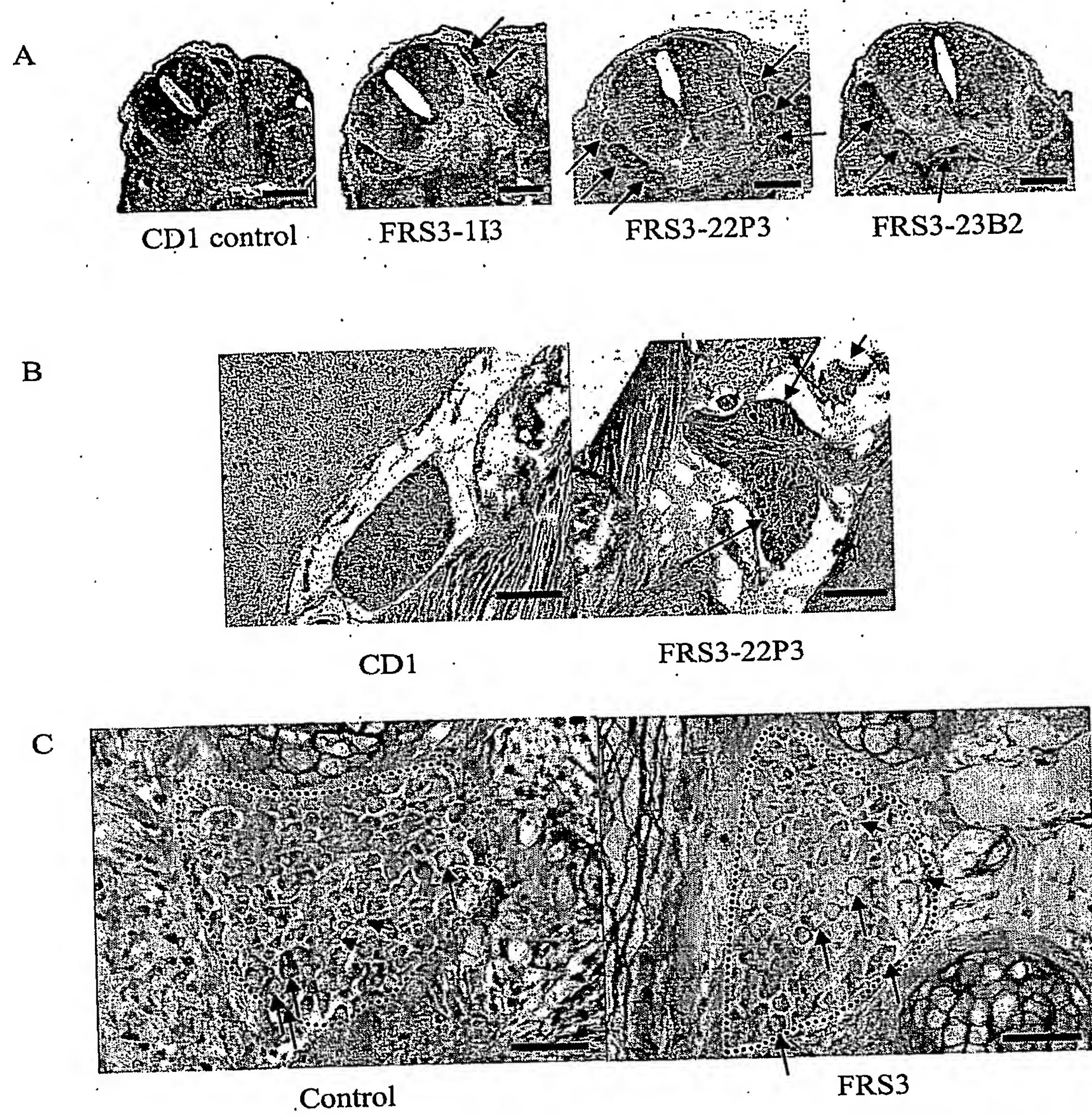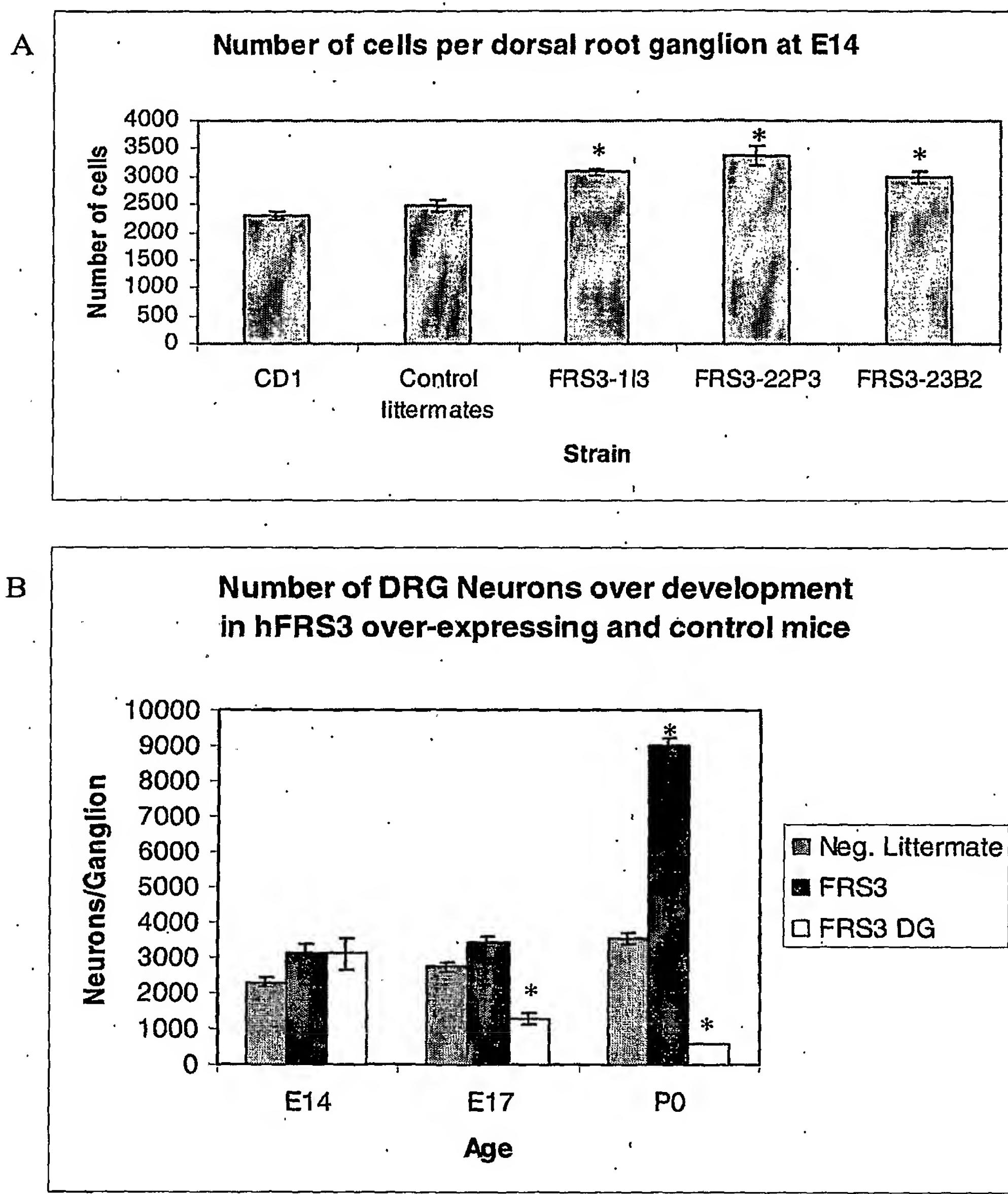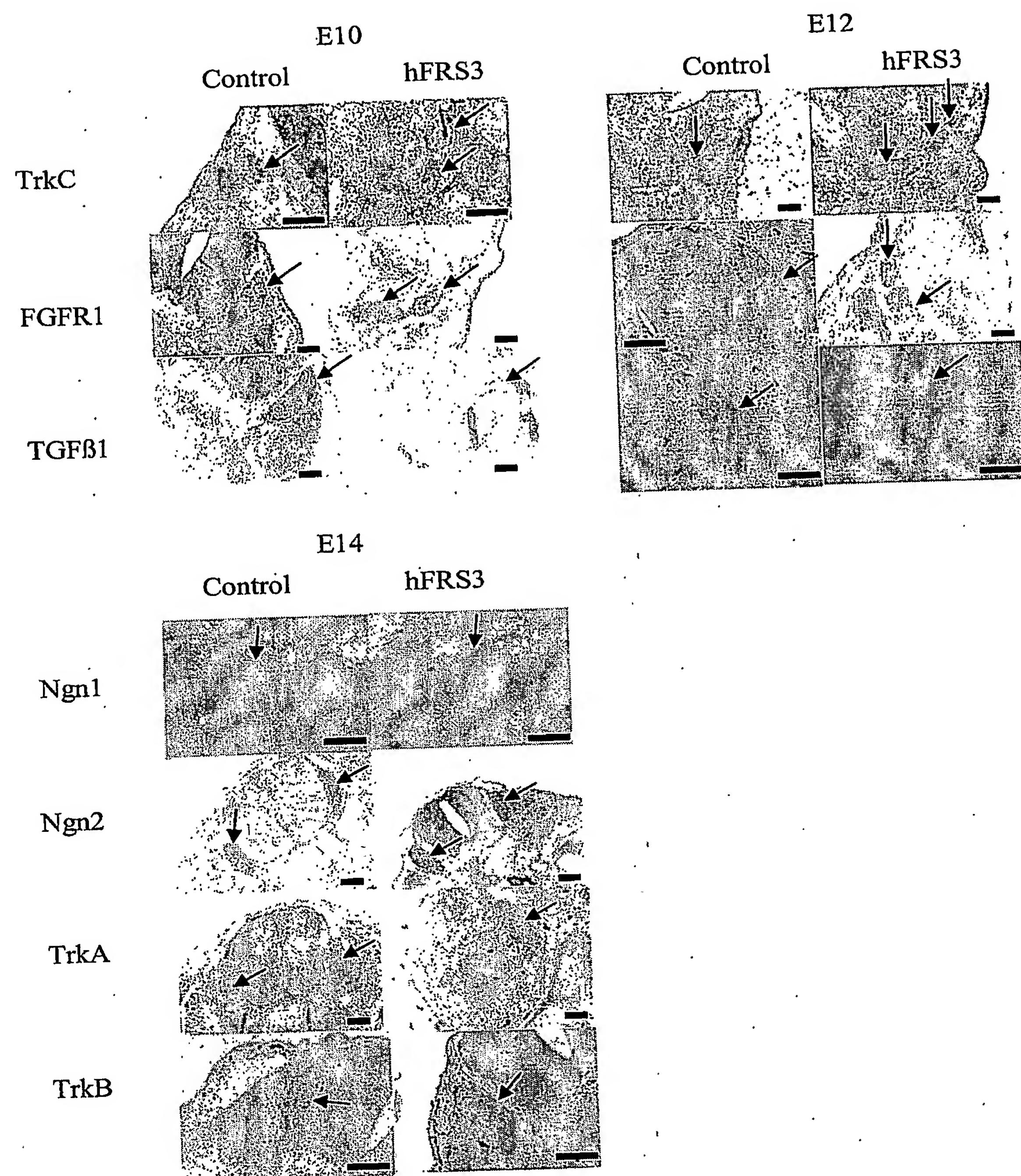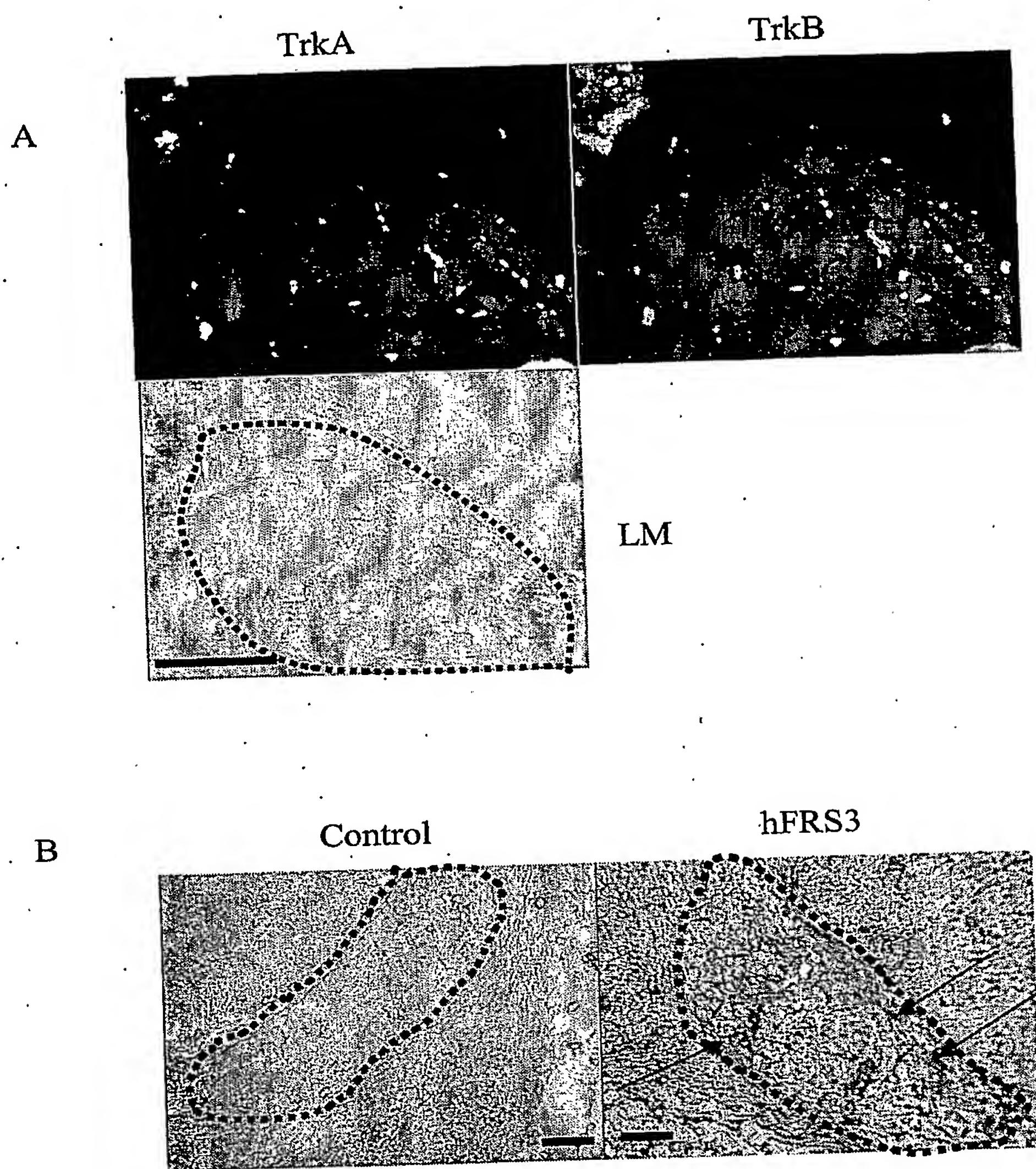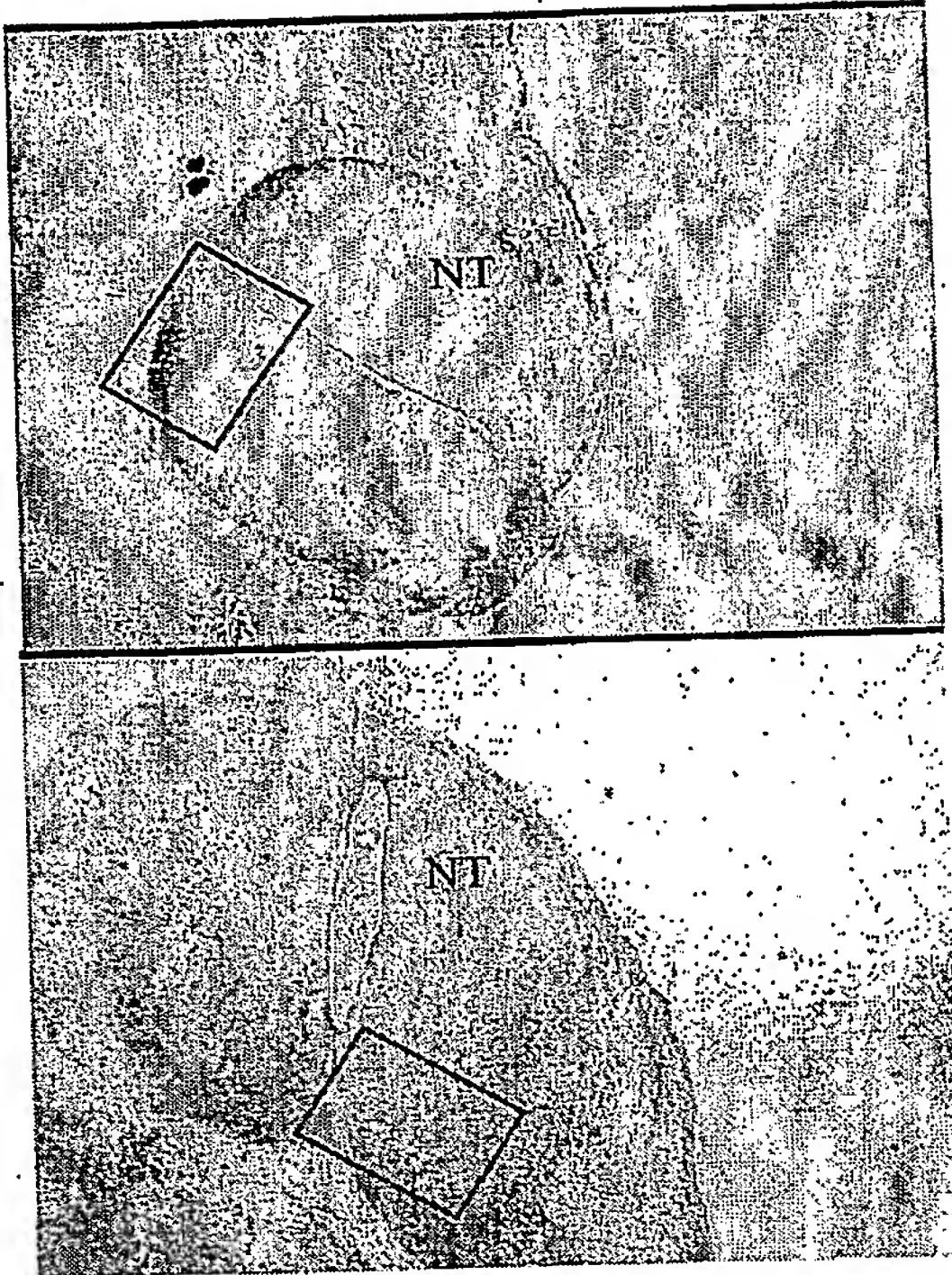
Figure 6

E12 neg.
littermates
Spinal cord
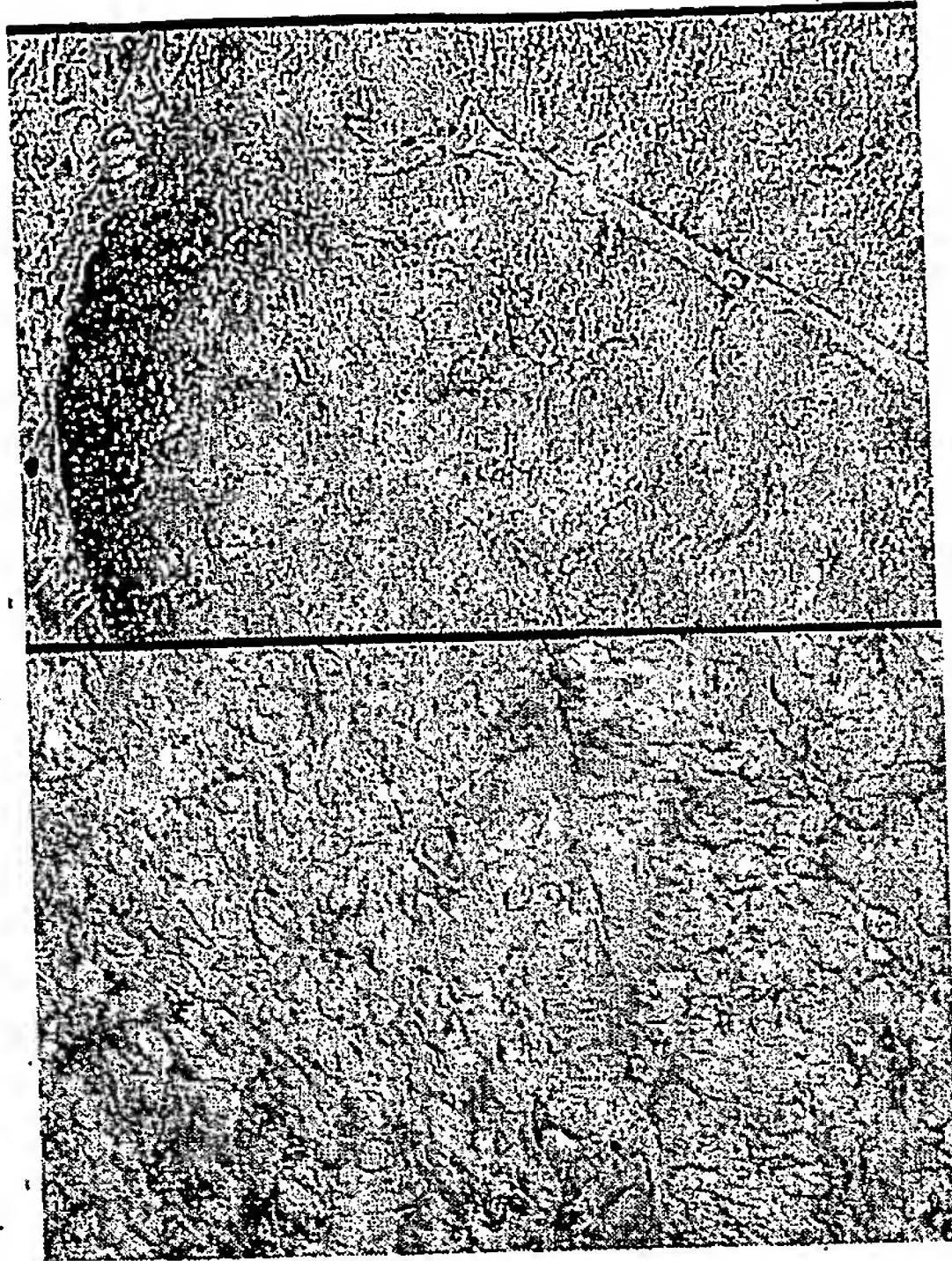
E12 FRS3
Spinal cord

Figure7

Figure 8

A

LacZ positive neural crest cells at 72 hours in culture

B

24h

72h
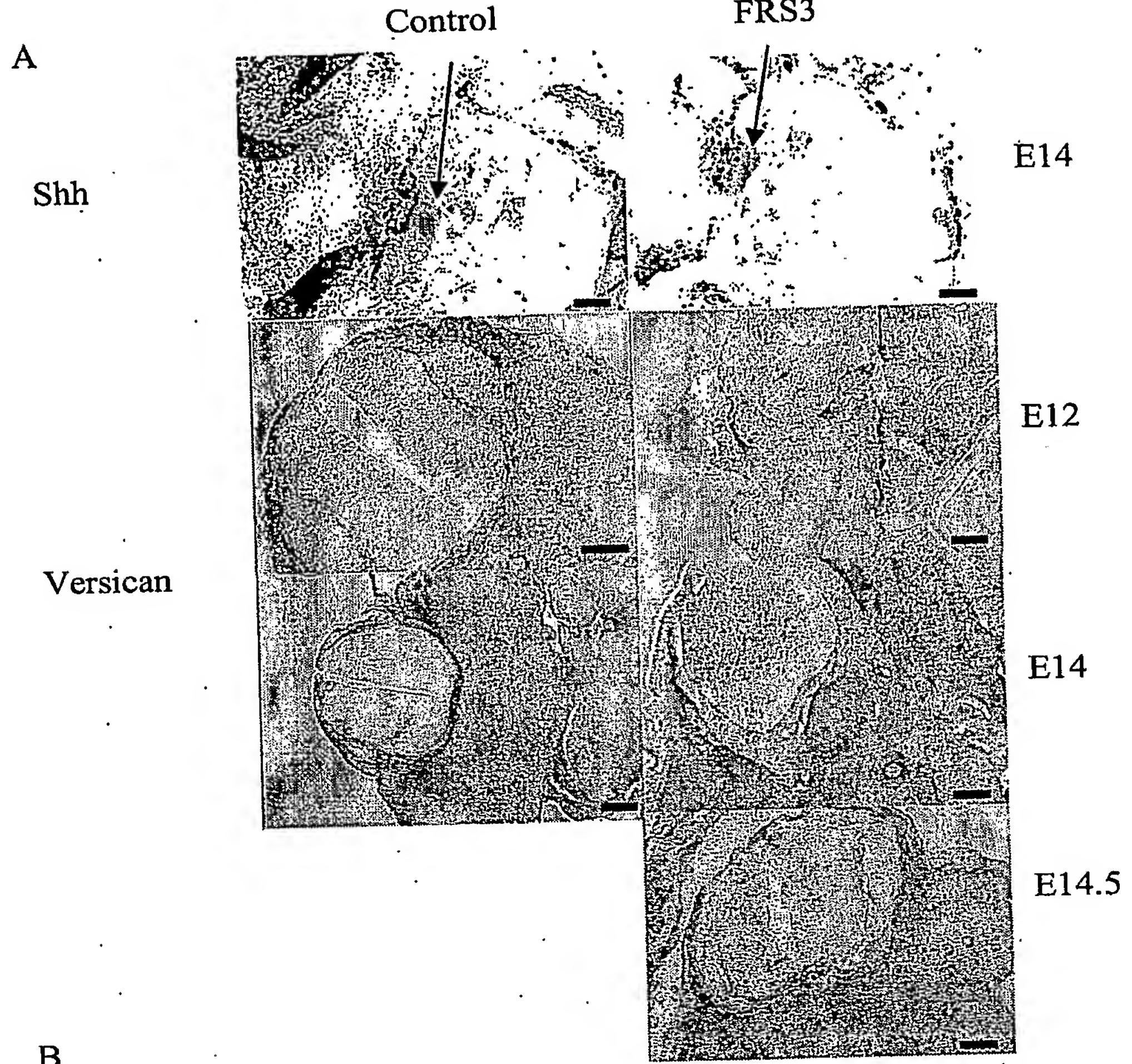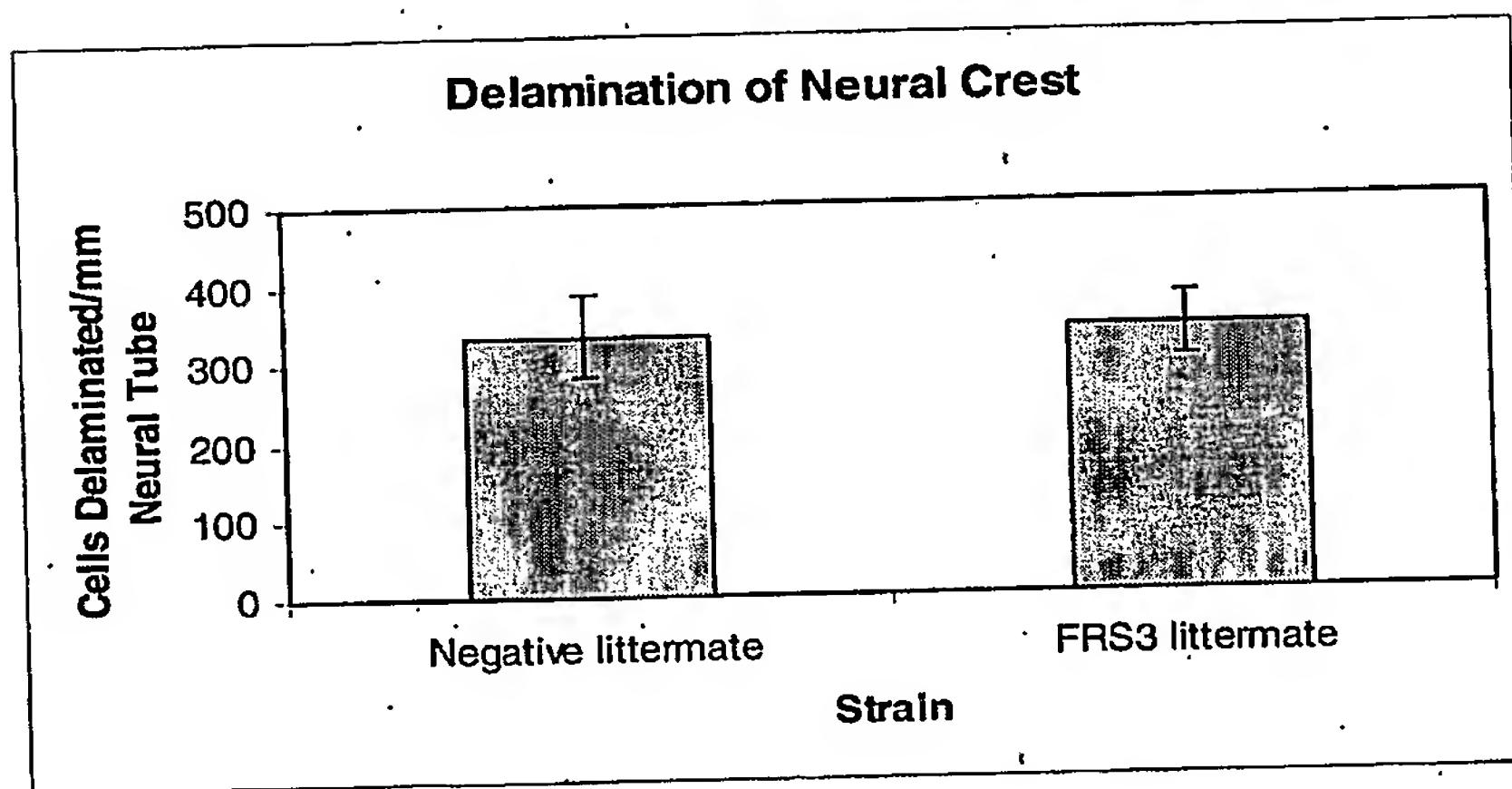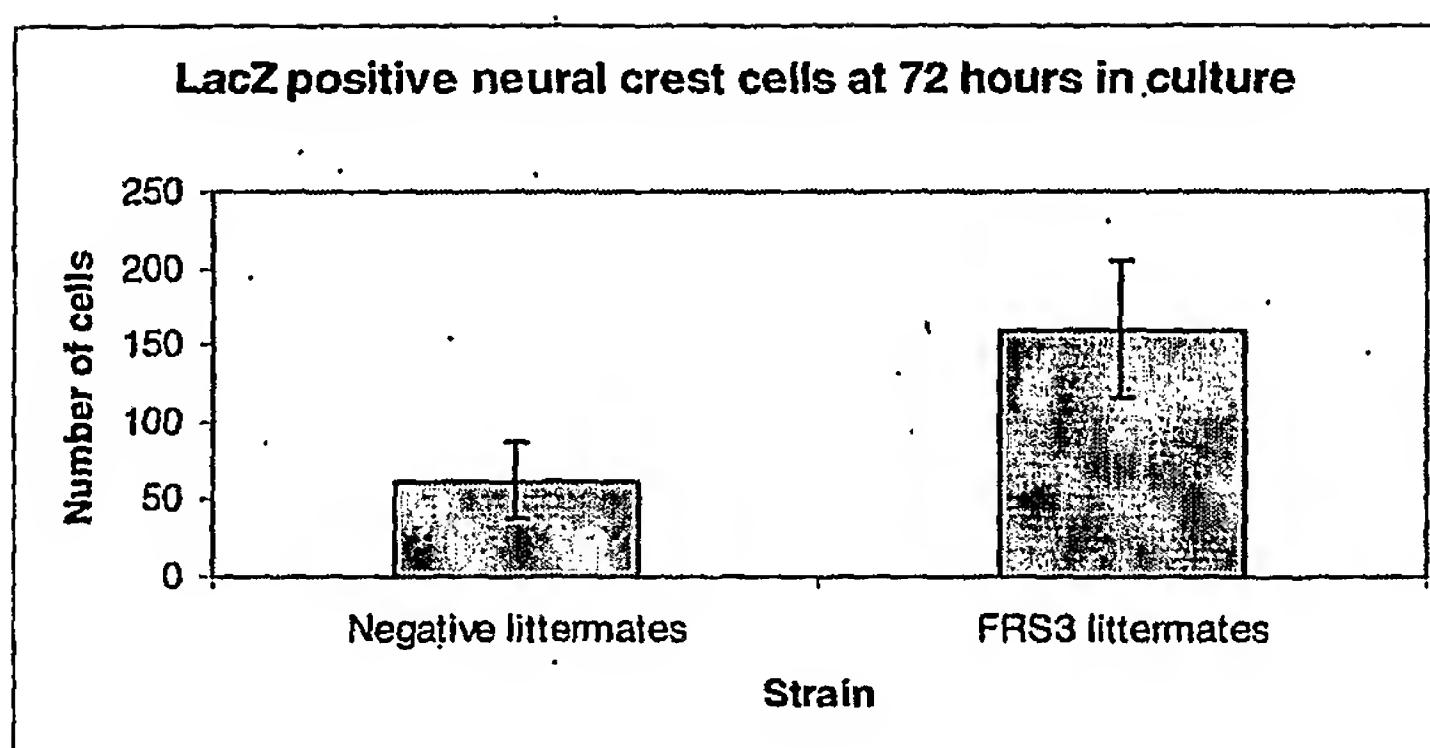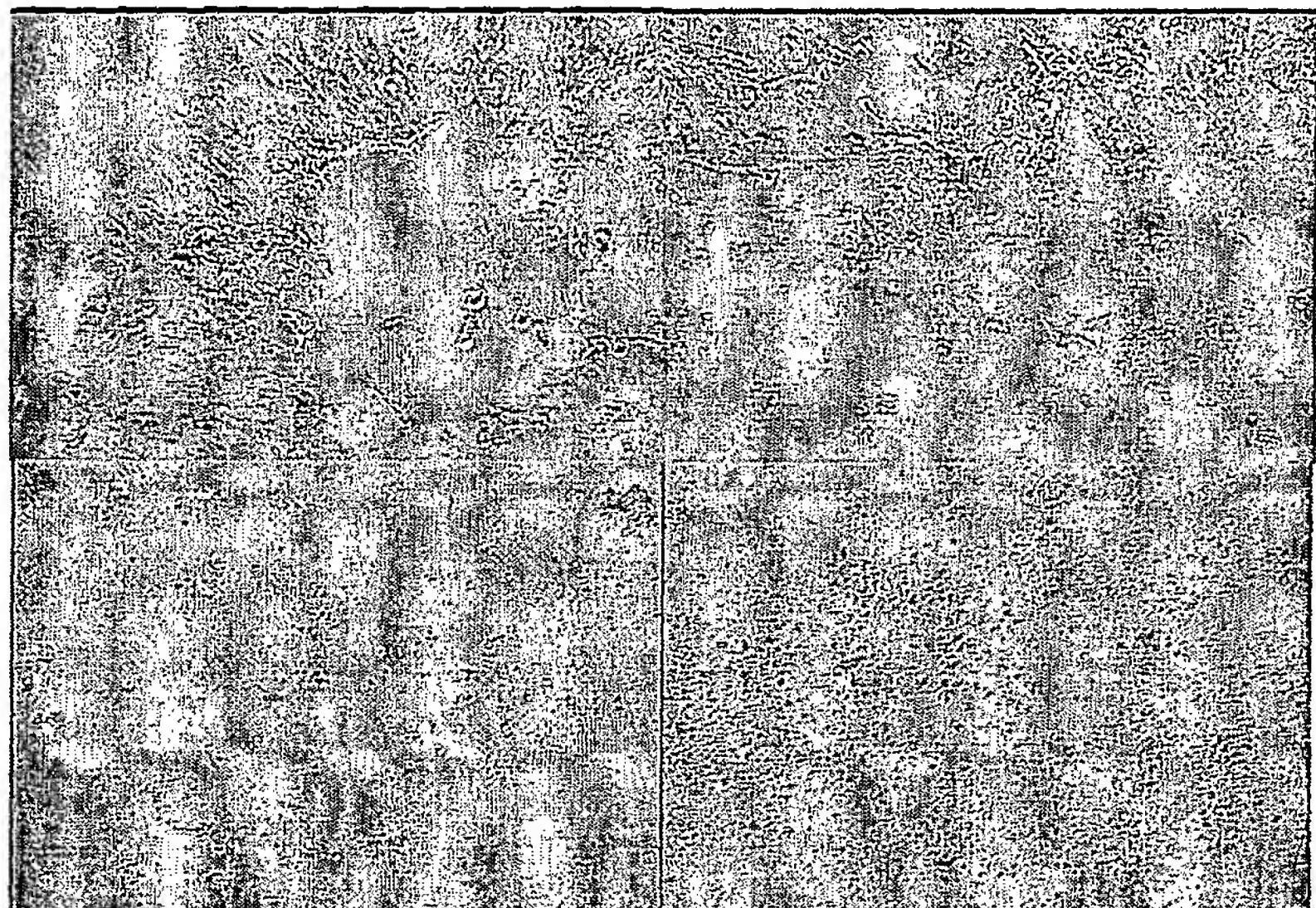
Negative littermate   FRS3

Figure 9